IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :
Tsuneo YASUMA et al. : Examiner: Chandrakumar, Nizal S
Serial No. 10/558,846 : Group Art Unit: 1625
Filed on November 30, 2005:
For : CONDENSED RING COMPOUND

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner for Patents
P.O.Box 1450
Alexandria, VA 22313-1450

Sirs:

I, Naoyuki Kanzaki, citizen of Japan and residing in Osaka-shi, Osaka-fu, Japan, sincerely declare;

That my education and employment history is as follows:

year	University/Company	Department	Job Title etc.	Research outline
1985	Osaka University, Graduate School of Engineering, Division of Fermentation Technology		Graduation (Master's degree)	
1985	Takeda Chemical Industry, Ltd. (now, Takeda Pharmaceutical Company, Ltd.)	Central Laboratories, Fermentation Product Laboratory	entrance	Engaged in research on culturing of inosine/guanosine-producing microorganism
1987		Food Laboratory		
1988		Applied Technology Laboratory		
1990		Food Vitamin Laboratory		Engaged in research on inosine/guanosine fermentation
1993		Food Vitamin Laboratory	Chief	Engaged in research on inosine/guanosine/beta-carotene fermentation

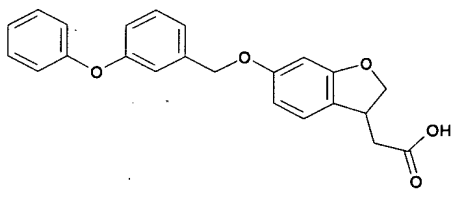
1994		Production Technology Laboratory, Center of Biotechnology	Scientist	Engaged in research on biotin fermentation
1995		Biotechnology Research Laboratory		Engaged in research on compound screening
1997			Assistant Research Head	
1998			Research Head	
1999		Pharmaceutical Developmental Research Division, Developmental 4th Laboratory		
2001-present		Pharmaceutical Research Division, Discovery Research Center		

That the following experiments were conducted under my supervision and direction in an attempt to show there are compounds having GPR40 agonistic activity but have no or little PPAR modulating activity.

Experiments

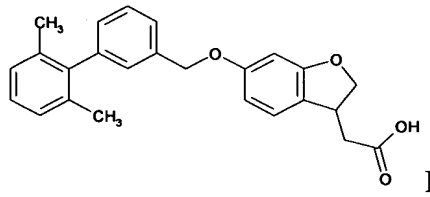
1. Object
   The purpose of the experiments are to show there are compounds having GPR40 agonistic activity but have no or little PPAR modulating activity.

2. Test Compound
   Compound A



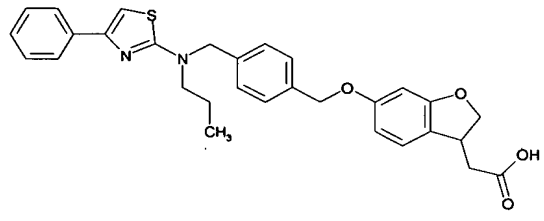
Example 31

Compound B



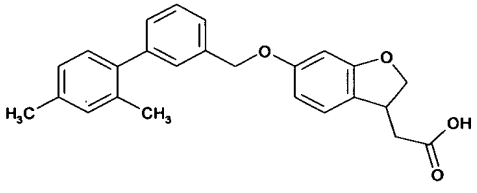
Example 33

Compound C



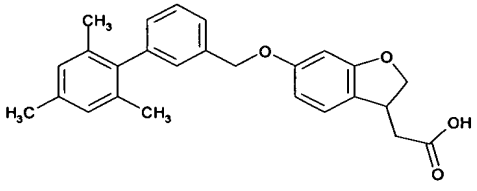
Example 35

Compound D



Example 37

Compound E



Example 39

All compounds described above are within the scope of the present claim.

3. Test Method

1) GPR40 agonistic activity

Using AlphaScreen cAMP assay kit (PerkinElmer), the amount of intracellular cAMP was quantitated. A CHO (dhfr-) cell expressing human GPR40 was cultivated with an MEM-α culture medium comprising 10% dialyzed serum (without nucleotide, Wako Pure Chemical Industries, Ltd. : 135-15175) at 37 °C until it became nearly confluent. The above-mentioned cells were washed with PBS (Invitrogen), and the cells were detached with 0.5 mM EDTA. HBSS (Invitrogen: 4065056) comprising 5 mM HEPES (pH 7.4) was added thereto to count

the number of cells. After centrifugation, the cells suspension was diluted with HBSS comprising 5 mM HEPES (pH 7.4) to make 2 x 10^6 cells/mL. The above-mentioned suspension (10 μL) was added to OptiPlate-384 (PerkinElmer) containing the diluted solution (final conc. 10 μM) of the test compound. The suspension was reacted at 37 °C for 30 min. After the reaction, the amount of intracellular cAMP was quantitated using AlphaScreen cAMP assay kit (PerkinElmer, 60625). More specifically, a solution of dispersed Anti-cAMP Acceptor beads was added to each well, and then a mixed solution of Biotinylated-cAMP, streptavidine beads Donor beads and Tween-20 was added to dissolve the cells. After 5 hours in room temperature, Envision (PerkinElmer) was used for fluorescent measurement (Ex: 680 nm, Em: 520-620) to obtain the generated cAMP concentration from the standard curve which was prepared separately.

2) PPAR modulating activity

PPAR reporter assay protocol

(1) Transactivation assay of hPPARγ1:

PPARγ:RXRα:PPRE×4/CHO-K1 cells were used for transactivation assays of hPPARγ1. These cells were seeded into an OPAQUE PLATE (white 96 well half area plate, COSTAR, U.S.A.) at the density of 1×10^4 cells/well, and cultured in 5% CO_2 at 37 °C overnight.

For the agonist assay, 45 μl of HAM F12 medium containing 0.1% fatty acid free-BSA and 5 μl of test compound (final conc. 10 μM) were added to the plate after removing the medium, and then the plate was cultured in 5% CO_2 at 37 °C for 1day.

Regarding the antagonist assay, 40 μl of HAM F12 medium containing 0.1% fatty acid free-BSA, 5 μl of test compounds and 5 μl of AD-5061(final conc. 10 nM), as known the potent PPARγ agonist, were added to the plate.

After 1day, the medium was removed from the incubated plate, and then 20 μl of PICAGENE-LT7.5 (Wako Pure Chemical Ind., Ltd., Japan), which was diluted to half with HANK'S BALANCED SALT SOLUTION, was added to each well. After stirring, luciferase activities were determined in microplate-based luminescence reader (Perkinnelmer, U.S.A.).

(2) Transient co-transfection assay of hPPARα and hPPARδ:

COS-1 cells were seeded at 5×10^6 cells in 150 cm^2 tissue culture flask, and cultured in 5% CO_2 at 37 °C overnight. Transfections were performed with LipofectAMINE (Life Technologies, Inc., U.S.A.) according to the instructions of manufacturer. Briefly, the

transfection mixture contained 125 µl of LipofectAMINE, 100 µl of plus reagent, 2.5 µg of each expression plasmid pMCMVneo-hPPARα (pMCMVneo-hPPARδ) and pMCMVneo-hRXRα, 5 µg of reporter plasmid pGL3-PPRE×4-tk-luc-neo, and 5 µg of pRL-tk (Promega, U.S.A.). Cells were incubated in 25 ml of transfection mixture for 3 h in 5% CO_2 at 37 °C. After adding 25 ml of DMEM medium (Life Technologies, Inc., U.S.A.) containing 0.1% fatty acid free-BSA, the cells were then incubated for 1day in 5% CO_2 at 37 °C. After transfection, cells were detached by treating with trypsin-EDTA (Life Technologies INC., U.S.A.) centrifuged and then suspended in DMEM medium containing 0.1% fatty acid free-BSA. The suspended cells were added into an OPAQUE PLATE (white 96 well half area plate, COSTAR, U.S.A.) at the density of 5×10^3 cells/well in 45 µl of DMEM medium containing 0.1% fatty acid free-BSA and 5 µl of test compound (final conc. 10 µM) and then cultured in 5% CO_2 at 37 °C for 2days. After removing the medium, 20 µl of PICAGENE-LT7.5 (Wako Pure Chemical Ind., Ltd., Japan), which was diluted to half with HANK'S BALANCED SALT SOLUTION, was added to each well. After stirring, luciferase activities were determined in microplate-based luminescence reader (Perkinnelmer, U.S.A.).

4. Results

The results are shown in the following Table 1.

Table 1

Compound	GPR40 agonistic activity at 10 µM (%)	PPAR α agonistic activity at 10 µM (%)	PPAR γ agonistic activity at 10 µM (%)	PPAR γ antagonistic activity against AD-5061 at 10 µM (%)	PPAR δ agonistic activity at 10 µM (%)
A	111	2	5	34	0
B	123	0	11	22	0
C	104	35	12	5	24
D	128	0	20	13	35
E	130	0	29	4	33

Control of agonistic activity (0% activity): without test compound

5. Conclusion

It has been evidenced that these claimed compounds having potent GPR40 agonistic activity, in spite they have no or little PPAR modulating activity.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at Osaka, Japan on this 8 day of October, 2008

Naoyuki Kanzaki

Naoyuki KANZAKI